

SPECIFICATION

METHOD OF CONSTRUCTING ANIMAL HAVING CANCER CELLS TRANSPLANTED THEREINTO

TECHNICAL FIELD

[0001] This invention relates to a process for preparing cancer cells transplanted animals in fields such as biology and medicine.

BACKGROUND ART

[0002] Cancer is the most common cause of death in Japan and it is said that about 30% of Japanese people die of cancer. In spite of the recent development of tailor-made medicine based on genomic information, therapeutics effective against cancer are yet to be discovered. Essential to the development of anti-cancer agents are appropriate cancer-bearing animals and their development is currently in need.

[0003] Cancer cells transplanted animals include knockout mice deprived of antioncogenes such as APC and p53, as well as animals in which cancer has been developed by various methods such as the use of chemicals and other carcinogenic agents and direct transplantation of cancer cells of interest. Among these animals, antioncogene knockout mice can be prepared in a fairly short period of time but, on the other hand, they are not easy to adopt since they are fairly expensive and subject to various limitations of organizations entrusted for commissioned production. Cancer development with chemicals requires a prolonged time to develop cancer, so much time is spent before a certain conclusion is reached.

[0004] Transplanting cancer cells has the advantage of giving experimental results in a short period of time. On the other hand, the transplanted cancer cells have poor "take" and the size and weight of the transplanted cancer tissue vary so greatly from one animal to another that evaluation of various anti-cancer agents involves difficulty in revealing any significant differences in their efficacy. Reasons for this defect include the poor "take" of the transplanted cancer cells and the leakage of the cancer cells suspension from the site of transplantation; it has therefore been necessary to improve the functions of the cells to be transplanted.

[0005] JP 05-192138 A describes a method of skin cells cultivation comprising the steps of preparing a cell culture support which has a surface of its base coated with a polymer having an upper or lower critical temperature for dissolution in water in a range of 0-80°C, cultivating skin cells on the cell culture support at a temperature not higher than the upper critical temperature for dissolution or at a temperature not lower than the lower critical temperature for dissolution, and thereafter adjusting the temperature to above the upper critical temperature for dissolution or below the lower critical temperature for dissolution, whereby the cultured skin cells are detached. This method depends on temperature adjustment for detaching the cells from the culture base coated with the temperature-responsive polymer; however, JP 05-192138 A neither describes nor suggests a method of preparing cancer cells transplanted animals using the thus obtained cells.

DISCLOSURE OF THE INVENTION

PROBLEMS TO BE SOLVED BY THE INVENTION

[0006] The present invention has been accomplished with a view to solving the aforementioned problems of the prior art. Thus, the present invention has as its object providing a new process for preparing cancer cells transplanted animals from an entirely different perspective than in the prior art.

MEANS FOR SOLVING THE PROBLEMS

[0007] In order to solve the aforementioned problems, the present inventors made R&D efforts based on a review from various viewpoints. They first prepared a cell culture support coated on a surface with a polymer the hydration force of which would change in a temperature range of 0-80°C; cancer cells were then cultivated on the support in a temperature region where the polymer had weak hydration force; thereafter, the culture solution was adjusted to a temperature at which the polymer had a stronger hydration force, whereby the cultured cancer cells were detached; the detached cancer cells were then transplanted to a specified site of an animal to be treated; surprisingly enough, this method turned out to be an efficient way of cancer cells transplantation. It was also found that the size and/or shape of the cancer tissue in the animal could be controlled by preparing a sheet of the cancer cells in a specified shape of a specified size. The present invention has been accomplished on the basis of these findings.

[0008] In general, the present invention provides a process

for preparing a cancer cells transplanted animal comprising the steps of preparing a cell culture support coated on a surface with a polymer the hydration force of which will change in a temperature range of 0-80°C, then cultivating cancer cells on the support in a temperature region where the polymer has weak hydration force, thereafter adjusting the culture solution to a temperature at which the polymer has a stronger hydration force, whereby the cultured cancer cells are detached, and transplanting the detached cancer cells to a specified site of an animal to be treated.

[0009] In a preferred embodiment of the process, a sheet of the cancer cells is prepared in a specified shape of a specified size so that the size and/or shape of the cancer tissue in the animal is controlled.

[0010] The present invention also provides a cancer cells transplanted animal prepared by the process described above.

[0011] The present invention further provides a method of selecting an anti-tumor agent comprising the steps of administering a test substance to an animal before and/or after transplanting cancer cells in the preparation process described above and evaluating the effect of the administered test substance on tumor formation.

ADVANTAGES OF THE INVENTION

[0012] In the process for preparing cancer cells transplanted animals described herein, the cultured cancer cells are detached without any enzyme treatment, so the adherent protein remains intact to allow for good "take" after

transplantation; as a further advantage, if a sheet of the cancer cells is prepared and applied to an animal, the leakage of the cancer cells suspension that would otherwise occur from the site of transplantation can be prevented to enable efficient preparation of a cancer cells transplanted animal.

BRIEF DESCRIPTION OF THE DRAWINGS

[0013] [Fig. 1] Fig. 1 is a graph showing how the tumor volume in nude mice that were transplanted with a sheet of cancer cells in their back in Example 1 changed over time, with the dimensions of a tumor being measured and its volume calculated for an ellipsoid;

[Fig. 2] Fig. 2 is a graph showing how the tumor volume in the same nude mice that were transplanted with a sheet of cancer cells in their back changed over time, with the dimensions of a tumor being measured and its volume calculated for a cylindroid;

[Fig. 3] Fig. 3 is a photo showing the back of a nude mouse before cancer cells transplantation;

[Fig. 4] Fig. 4 is a photo showing the back of a nude mouse 4 weeks after transplanting a sheet of cancer cells; and

[Fig. 5] Fig. 5 is a photo showing the back of a nude mouse 4 weeks after transplanting a suspension of cancer cells.

BEST MODE FOR CARRYING OUT THE INVENTION

[0014] The cells to be used in the present invention may be any cancer cells that are directly collected from a living

tissue; alternatively, they include, but are not limited to, cell lines such as HBC-4, BSY-1, HBC-5, MCF-5, MCF-7, MDA-MB-231, U251, SF-268, SF-295, SF-539, SNB-75, SNB-78, HCC2998, KM-12, HT-29, WiDr, HCT-15, HCT-116, NCI-H23, NCI-H226, NIC-H522, NCI-H460, A549, DMS273, DMS114, LOX-IMVI, OVCAR-3, OVCAR-4, OVCAR-5, OVCAR-8, SK-OV-3, RXF-631L, ACHN, St-4, MKN1, MKN7, MKN28, MKN45, and MKN74. Cell lines known to be "untransplantable" such as MGF-40, MGT-90, CS-C9 and CS-C20 can also be transplanted with high percent take by employing the technique of the present invention. The cells to be used in the invention can be derived from various sources that include but are by no means limited to human being, dog, cat, rabbit, rat, swine and sheep. If the cultured cells of the present invention are to be used in the treatment of humans, human-derived cells are preferably used. The culture medium for cultivating cells in the present invention is not limited in any particular way as long as it is conventionally used with the cells to be cultivated.

[0015] In the present invention, the cells described above are cultivated on a cell culture support coated on a surface with a polymer the hydration force of which changes in a temperature range of 0-80°C, the cultivation temperature being in a region where the polymer has weak hydration force. The temperature region where the polymer has weak hydration force is by no means limited as long as the polymer coating is in a desolvated state and the applicable temperature is typically in the cell cultivating temperature range of 35-38°C, with 37°C being particularly preferred. Cells will adhere and grow if

the cell culture support is coated with the polymer in the amount to be described below and if the polymer remains dehydrated. The polymer to be used in the present invention is of such a nature that its hydration force changes abruptly at a temperature characteristic of it in the range of 0-80°C. To be more specific, the polymer makes a sudden shift from a dehydrated to a hydrated state at that characteristic temperature. Being coated with this polymer, the surface of the cell culture support material on which the cells have adhered and grown changes to a non-adherent state, making it possible to detach the cultured cells. According to the present invention, there is no need to use enzymes such as trypsin but one may simply change the cultivation temperature to detach the cultured cells; hence, the detached cell sheet is only lightly damaged, free from the damage it would receive if it were treated with an enzyme such as trypsin. Since the detachment of the cultured cancer cells involves no enzyme treatment, the adherent protein remains intact, assuring good "take" after transplantation; if the cancer cells are in a sheet form, there is another advantage in that the leakage of a cell suspension from the site of transplantation is effectively suppressed to allow for efficient preparation of a cancer cells transplanted animal.

[0016] The temperature-responsive polymer to be used in the present invention may be a homo- or copolymer. Examples of such polymer are described in JP 2-211865 A. Specifically, the intended polymer can be obtained by homo- or copolymerization of the monomers listed below. Applicable

monomers include (meth)acrylamide compounds, N- (or N,N-di)alkyl-substituted (meth)acrylamide derivatives, and vinyl ether derivatives. In the case of copolymers, any two or more of these monomers may be employed. If desired, they may be copolymerized with other monomers, or the resulting polymers may be subjected to graft polymerization or copolymerization, or mixtures of polymers and copolymers may be employed. The polymers can also be crosslinked to the extent that will not impair their inherent properties. The method of coating the base surface with the various polymers mentioned above is not limited in any particular way and an exemplary method that can be followed is described in JP 2-211865 A. To be specific, coating can be done by subjecting the base and the monomers or polymers mentioned above to irradiation with electron beams (EB), γ -rays or ultraviolet light, or plasma or corona treatment, or organic polymerization reaction; alternatively, physical adsorption can be effected by coating or blending. The amount in which hydrophilic polymers are to be immobilized at the site of cell adhesion is not limited to any particular value as long as they are sufficient to adhere the cells that need to be moved; however, since cancer cells are used in the present invention, the hydrophilic polymers are typically immobilized in an amount of at least $0.4 \mu\text{g}/\text{cm}^2$, preferably at least $0.8 \mu\text{g}/\text{cm}^2$, and more preferably at least $1.2 \mu\text{g}/\text{cm}^2$. The amount of polymer immobilization may be measured by the usual method; in one example, the site of cell adhesion is directly measured by FT-IR-ATR, and in another example, a preliminarily labeled polymer is first immobilized at the site of cell

adhesion by the same method and the amount of interest is estimated from the amount of the immobilized labeled polymer. Either of these methods is practically feasible.

[0017] The shape of the culture base to be used in the present invention is not limited in any particular way; examples include shapes such as a dish, a multi-well plate, a flask and a cell insert, as well as a flat membrane. The base to be coated with the polymer is one that is customarily used in cell culture and may be exemplified by glass, modified glass, compounds such as polystyrene and poly(methyl methacrylate), and all those materials that generally can be given a shape, including high-molecular weight compounds and ceramics other than those mentioned above.

[0018] The temperature-responsive polymer with which the base of the cell culture support is coated for use in the present invention tends to undergo hydration or dehydration in response to a change in temperature within a certain region, which has turned out to range from 0°C to 80°C, preferably from 10°C to 50°C, and more preferably from 20°C to 45°C. Temperatures beyond 80°C are not preferred since cancer cells are prone to die. Temperatures lower than 0°C are also not preferred since the cell growth rate drops considerably or the cancer cells die.

[0019] In order to detach and recover the cultured cancer cells from the support material in the present invention, they are optionally brought into intimate contact with a carrier and then the support material to which the cells adhering is adjusted to a temperature at which the polymer coat on the

support base undergoes hydration, whereupon the cancer cells can be detached intact from the support together with the carrier. In this case, a water stream may be applied between the cell sheet and the support assure smooth detachment. The detachment of the cell sheet may be effected within the culture solution in which the cells have been cultivated or in other isotonic solutions, whichever is suitable for a specific purpose.

[0020] The cancer cells cultivated in the present invention are free from the damage which they would sustain if they were treated with proteolytic enzymes represented by dispase and trypsin. Therefore, the cancer cells detached from the base have an adherent protein and if they are detached in a sheet form, the desmosome structure between cells will be retained to some extent. As a result, the detached cancer cells can adhere satisfactorily to the diseased tissue to which they have been transplanted, thus assuring efficient transplantation. It is generally known that cells treated with the proteolytic enzyme dispase can be detached while retaining 10-60% of the desmosome structure between cells but, on the other hand, most of the basement membrane-like protein between cell and the base is destroyed, with the detached cell sheet having only low strength. In contrast, the cancer cells sheet prepared in the present invention keeps both the desmosome structure and the basement membrane-like protein intact in a respective amount of at least 80%, thereby providing the various advantages already described above.

[0021] The foregoing description is paraphrased below by

referring to poly(N-isopropylacrylamide) as an example of the temperature-responsive polymer. Poly(N-isopropylacrylamide) is known as a polymer having a lower critical temperature for dissolution at 31°C and if it is in a free state, it undergoes dehydration in water at a temperature above 31°C, with the polymer chains agglomerating to cause turbidity. Conversely, at 31°C and below, the polymer chains become hydrated and dissolve in water. In the present invention, a coat of this polymer is immobilized on a surface of the base such as a Petri dish. Therefore, at a temperature above 31°C, the polymer on the base surface likewise undergoes dehydration but with the polymer chains being immobilized to form a coat on the base surface, the latter comes to show hydrophobicity; on the other hand, at 31°C and below, the polymer on the base surface undergoes hydration but with the coat of polymer chains being immobilized on the base surface, the latter comes to show hydrophilicity. The hydrophobic surface is appropriate for the adhesion and growth of cells whereas the hydrophilic surface hates cell adhesion so much that the cells being cultivated or the cell sheet needs only to be cooled to become detached.

[0022] The carrier to be used for bringing the cancer cells or the sheet of cancer cells into intimate contact with the culture base is a structure for holding the cells used in the present invention and examples that can be used include high-molecular weight membranes, structures shaped from high-molecular weight membranes, and metallic jigs. If a high-molecular weight membrane is to be used as a material for the

carrier, specific examples include poly(vinylidene difluoride) [PVDF], polypropylene, polyethylene, cellulose or its derivatives, paper and like products, chitin, chitosan, collagen, and polyurethane. The shape of the carrier is not limited in any particular way.

[0023] The cancer cells to be transplanted in the present invention have good "take", so they only need to be transplanted in a total number of 1×10^5 cells or less, preferably 5×10^5 cells or less, and more preferably 8×10^5 or less. In the case of the present invention, transplanting more than 8×10^5 cancer cells is advantageous since a large enough cancer tissue can be obtained but, on the other hand, an undesirably large number of cells have to be handled at a time. The site of transplantation is not limited at all and cancer cells may be transplanted under the skin or they may be directly transplanted to the tissue derived from specific cancer cells.

[0024] Animals that can be used as recipients for transplantation in the present invention include, but are not limited to, nude mouse, rat, mouse, guinea pig, and rabbit.

[0025] As described above, the high-take cancer cells to be used in the present invention can adhere very satisfactorily to living tissues to ensure the preparation of cancer cells transplanted animals within a very short period of time that has been quite impossible to realize in the prior art.

[0026] The cancer cells transplanted animal prepared in the present invention can be employed in a method of selecting an anti-tumor agent comprises the steps of administering a test

substance to an animal before and/or after transplanting cancer cells in the preparation process and evaluating the effect of the administered test substance on tumor formation.

Examples

The present invention is described below in greater detail with reference to examples which are by no means intended to limit the scope of the present invention.

Example 1

[0027] A cell culture base was coated with the temperature-responsive polymer poly(N-isopropylacrylamide) in an amount of $2.0 \mu\text{g}/\text{cm}^2$ and the cancer cells NCI-H460 was cultivated (2×10^4 cells were seeded; 37°C in 5% CO_2). Three days later, the cancer cells (NCI-H460) on the culture base were confirmed to have become confluent; thereafter, a cultured cell moving jig comprising a polyacrylic plate coated with a fibrin gel was gently placed over the cultured cell sheet so that the cultured cancer cells adhered to it; then, the cell culture base was cooled at 20°C for 60 minutes. After the cooling, the detached cell sheet was collected from the jig together with the fibrin gel and a piece of the gel with the adhering cell sheet (7 mm x 17 mm x 2 mm; 5×10^5 cells) was transplanted subcutaneously to the back of each of 10 nude mice. The dimensions of the tumor that developed after the transplantation were measured over the skin with a micrometer and the results obtained by calculating the volume of the tumor assuming that it was an ellipsoid are shown in Fig. 1 (volume of ellipsoid = $\pi/6 \times \text{major axis of the tumor} \times \text{minor}$

axis of the tumor x thickness of the tumor). The results obtained by calculating the volume of the tumor assuming that it was a cylindroid are shown in Fig. 2 (volume of cylindroid = $\pi/4$ x major axis of the tumor x minor axis of the tumor x thickness of the tumor). Four weeks after the transplantation, the mean volume of ellipsoid was $581.7 \pm 566.3 \text{ mm}^3$, the mean volume of cylindroid was $1302.7 \pm 1007.9 \text{ mm}^3$, and the mean tumor weight was $776.9 \pm 534 \text{ mg}$. Fig. 3 is a photo showing the back of a nude mouse before the transplantation and Fig. 4 is a photo showing the back of the same nude mouse 4 weeks after transplanting the cancer cells sheet.

Comparative Example 1

[0028] The cancer cells NCI-H460 was cultivated on a cell culture base with no temperature-responsive polymer coat on its surface (2×10^4 cells were seeded; 37°C in 5% CO_2). Three days later, the cancer cells (NCI-H460) on the culture base were confirmed to have become confluent; thereafter, trypsin treatment was performed to recover the cancer cells. A suspension of the recovered cancer cells (5×10^5) was transplanted subcutaneously to the back of each of two nude mice. As in Example 1, the tumor volume was calculated on the assumption that it was either an ellipsoid or a cylindroid, and the respective results are also shown in Figs. 1 and 2. Four weeks after the transplantation, the mean volume of ellipsoid was 40.7 mm^3 , the mean volume of cylindroid was 60.7 mm^3 , and the mean tumor weight was 74.2 mg . Fig. 5 is a photo showing the back of a nude mouse 4 weeks after

transplanting the suspension of cancer cells.

Example 2

[0029] A cell culture base was coated with the temperature-responsive polymer poly(N-isopropylacrylamide) in an amount of $1.9 \mu\text{g}/\text{cm}^2$ and the cancer cells A-549 was cultivated (2×10^4 cells were seeded; 37°C in 5% CO_2). Three days later, the cancer cells (A-549) on the culture base were confirmed to have become confluent; thereafter, a poly(vinylidene difluoride) [PVDF] membrane not coated with a fibrin gel was gently placed over the cultured cell sheet so that the cultured cancer cells adhered to it; then, the cell culture base was cooled at 20°C for 60 minutes. After the cooling, a sheet of cancer cells (7 mm x 17 mm x 2 mm; 5×10^5 cells) was detached together with PVDF membrane. The back of each of 10 nude mice was incised linearly beneath the skin and the subcutaneous tissue was detached with forceps to create a pocket, into which the above-described cancer cells sheet was inserted. After the inserting, the incised part was sutured to complete the transplantation. The dimensions of the tumor that developed after the transplantation were measured over the skin with a micrometer and the volume of the tumor was calculated assuming that it was an ellipsoid. The volume of the tumor was also calculated, this time on the assumption that it was a cylindroid (volume of cylindroid = $\pi/4 \times$ major axis of the tumor x minor axis of the tumor x thickness of the tumor). Four weeks after the transplantation, the mean volume of ellipsoid was $578.7 \pm 322.8 \text{ mm}^3$, the mean volume of cylindroid was $1258.7 \pm 897.9 \text{ mm}^3$, and the mean tumor weight

was 785.4 ± 394 mg. The decrease in the tumor volume that occurred immediately after the transplantation as shown in Fig. 1 (tumor assumed as an ellipsoid) and Fig. 2 (as a cylindroid) was absent and the tumor grew bigger as more days elapsed after the transplantation.

Example 3

[0030] The cancer cells transplanted animals prepared in Example 2 were administered a total of four times on a once-a-week basis with 3 mg of 5-fluorouracil (5-FU), a known anti-tumor agent, through the tail vein as it was dissolved in 0.3 ml of 1% ethyl alcohol containing physiological saline. Four weeks after the administration, the mean volume of ellipsoid was 279.6 ± 127.1 mm³, the mean volume of cylindroid was 619.3 ± 262.9 mm³, and the mean tumor weight was 369.3 ± 123 mg. As it turned out, the cancer cells transplanted animals described in the present invention got the volume and weight of the tumor to be reduced by receiving the anti-tumor agent. Obviously, the cancer cells transplanted animals of the present invention are useful in selecting an effective anti-tumor agent.

INDUSTRIAL APPLICABILITY

[0031] According to the process of the present invention, cultured cancer cells can be conveniently detached without using any proteolytic enzyme and cancer cells transplanted animals can be prepared efficiently.